The Bioavailability of Astaxanthin Is Dependent on Both the Source and the Isomeric Variants of the Molecule

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Abstract

Astaxanthin is a marine carotenoid that has a number of potential health benefits, including a very strong antioxidant potential. Present in the flesh of salmonids and shellfish, its natural sources currently on the market for food supplements come from the algae *Haematococcus pluvialis* and krill. However other natural sources can be found and may be of interest.Cellular uptake studies were performed on Caco-2/TC7 colonic cells. The cells were cultured on a semi-permeable membrane to create a polarized and functional epithelium, representative of the intestinal barrier. Four sources of astaxanthin were selected and compared; synthetic, natural extracts from bacteria, algae or yeast. Astaxanthin was incorporated at a concentration of 5µM into mixed micelles and applied to cultured cellsand concentration of astaxanthin measured by HPLC in both apical and basolateral compartments. Small variations in bioavailability were observed at 3 hours. After 6 hours, only the algae source of astaxanthin was still present in the apical compartment as the esterified form. Structure-activity relationships are further discussed. Animal experiments usingyeast and algae sources in different types of matrices confirm the role of source and formulation in the bioavailability potential of astaxanthin.

Keywords: *astaxanthin,bioavailability, Caco-2 cells, carotenoids, lipid metabolism*

INTRODUCTION

Astaxanthin belongs to the family of carotenoids and has powerful antioxidant properties. Increasing interest has been targeted toward this molecule and its biological benefits in recent decades. Several studies compared the antioxidant activity of astaxanthin with other carotenoids. One study found that astaxanthin neutralized twice as efficiently singlet oxygen as beta-carotene (and almost 80 times more effective than vitamin E) in chemical solution (Mascio *et al.* , 1991). Lycopene by comparison was 30% more efficient than astaxanthin. Similar results were observed by researchers working on in vitro system of human blood cells treated with various carotenoids and then exposed to singlet oxygen. Again, lycopene has proved the most effective, followed by zeaxanthin and beta-carotene (Tinkler *et al.*, 1994). Astaxanthin also neutralizes free radicals. A study shows that it is 50 times more effective in preventing peroxidation of fatty acids that beta-carotene or zeaxanthin(Terao, 1989). In most aquatic animals in which it can be found, astaxanthin has several essential biological functions, including protection against the oxidation of polyunsaturated essential fatty acids protect against the effects of UV light, immune response modulation, pigmentation, communication, reproductive behaviour and the improvement of reproduction (Lorenz &Cysewski, 2000).

Astaxanthin cannot be synthesized by most animals and must be acquired from the diet. Although

mammals and most fish are unable to convert other dietary carotenoids to astaxanthin, crustaceans (such as shrimp) and some fish species (koi) have a limited ability to convert closely related dietary carotenoids into astaxanthin, although most astaxanthin recovered comes from their diet. Mammals do not have the capacity to synthesize astaxanthin and unlike beta-carotene astaxanthin has no provitamin A activity in these animals.

Astaxanthin is naturally present in the human diet with seafood such as krill, shrimp, lobster, cod, mackerel, salmon or other coloured fish. In wild salmon, concentrations of astaxanthin can reach 40 mg/kg while farmed salmon can only reach up to 8 mg/kg(Ambati*et al.*, 2014). The daily intake of 2-4 mg astaxanthin, recommended for physiological effects, thus corresponds to the absorption of 100 g of wild salmon or 500 g of farmed salmon. The main non-animal natural sources of astaxanthin are the microalgae *Haematococcus pluvialis* and yeast *Phaffia rhodozyma*. Only algae astaxanthin is currently marketed as dietary supplement for humans; *Phaffia rhodozyma* yeast extracts on the other hand are currently only used for animal feed (fish, eggs). The synthetic source is also widely used in animal feed, while production by the bacterium *Paracoccuscarotinifaciens* is more anecdotal. These four sources present specific geometric and optical isomers detailed in Tab. 1.The astaxanthin profile being identical in fish flesh to that present in their diet (Storebakken *et al.*, 1985), the different forms used in aquaculture therefore find themselves indirectly in the human diet.

The various stages of transportation, digestion, absorption and transport in the plasma of dietary carotenoids were examined in mammals (Furr& Clark, 1997)but also because these compounds have been associated with reducing risks of certain cancers and chronic diseases. Full understanding of carotenoid metabolism is complicated by a number of factors: variations in physiochemical properties among carotenoids; altered carotenoid utilization as a result of the normal vicissitudes of lipid absorption and transport; divergence in metabolic fate within the intestinal enterocyte (especially carotenoid cleavage to retinoids. In plasma, the non-polar carotenoids such as beta-carotene, alpha-carotene or lycopene are usually transported by very low density lipoproteins (VLDL) and low density lipoproteins (LDL) and the polar carotenoids such as zeaxanthin, lutein or astaxanthin are more likely to be transported by LDL and high density lipoprotein (HDL). Similarly, a limited number of clinical studies have investigated the bioavailability of astaxanthin from algae (Mercke-Odeberg*et al.*, 2003; Okada, Ishikura, &Maoka, 2009) or a synthetic esterified form (Coral-Hinostroza*et al.*, 2004; Østerlie *et al.*, 2000)pharmacokinetics, and distribution of astaxanthin E/Z and R/S isomers in plasma and lipoprotein fractions were studied in 3 middle-aged male volunteers (37-43 years but none so far on fermentative sources such as yeast or bacteria.

To our knowledge, no report comparing the different sources of astaxanthin has been published. This study therefore aims to compare on a cellular epithelial transport model four sources of astaxanthin used in animal feed, some of which are used or intended for human consumption.

MATERIALS AND METHODS

All reagents were purchased from Sigma Aldrich (France), except for the different astaxanthin extracts which were generously donated by Ajinomoto Foods Europe, Algatechnologies ltd and ACS Dobfar spa for the yeast, algae and bacterial sources, respectively. We followed an existing protocol (Mimoun-Benarroch *et al.*, 2011) to mimic the intestinal absorption of lipophilic molecules solubilized in the form of bile salt micelles. This formulation, based on the composition of postprandial duodenal contents in humans (Armand *et al.*, 1996), appears to be the closest to physiological conditions. In brief, cholesterol (0.1 mM), phosphatidyl choline (0.5 mM), lyso-phosphatidyl choline (1.5 mM), monoolein (0.03 mM), sodium oleate (0.5 mM) and astaxanthin (5 µM) were mixed in a methanol:chloroform solution (2/1; v/v) and evaporated under a stream of nitrogen. Sodium taurocholate (5 mM) was then diluted in half a volume of culture medium (DMEM) without serum and without phenol red and added to the dried lipid residue and vigorously mixed by sonication at 25 W for 3 min. When the medium is translucent, the second volume of culture medium is added and the solution stirred overnight to allow the micelles to stabilize. The solution is filtered (0.2 µm) before treatment to retain only the uniform size of micelles.

Media and cell culture solutions were purchased from Life Technologies (France). Caco2/TC7 cellswere a generous gift from Dr Monique

Tab. 1. Sources of astaxanthin (AX) and their structural variations

Source	AX (ppm)	Optical isomers (%)			Geometrical isomers (%)		Derivation (%)		
		3R,3'R	3R,3'S	3S,3'S	All-trans	Cis	Free	Mono-ester	Diester
farmed salmon	2.5-8	25	50	25					
wild salmon	5-30	12-17	2-6	78-85					
krill	120	9-55	7-21	38-70	75	25	4-5	31-35	61-64
Phaffia rhodozyma	5000-10000	98	-	2	70-90	10-30	100	-	-
Paracoccuscarotinifaciens	20000	-	-	100	95	5	100	-	-
Haematococcus pluvialis	10000-40000	4	8	88	70	30	5	85	15
Synthetic	100000	25	50	25	65-75	25-35	100	-	-

Rousset (Université Pierre et Marie Curie-Paris 6, UMR S872, Les Cordeliers, Paris). They were cultured in DMEM Glutamax medium, 4.5 g/L glucose, 1 % antibiotics, 1 % nonessential amino acids, 20 % inactivated foetal calf serum, in an incubator at 37 °C and 10 % CO_2. To mimic the enterocyte transport, the cells were seeded at a density of 0.25 × 10^6 cells in inserts containing a semi permeable PET membrane (23.1 mm in diameter; 1 µm porosity) placed in 6-well plates (Becton Dickinson). The use of inserts allows differential access to the two poles of the cell, the apical compartment representing the intestinal lumen and basolateral compartment representing the internal circulation. In brief, cells were cultured with complete medium (20% serum) for 7 days after seeding until it formed a compact cell monolayer, as validated by transepithelial electrical resistance (TEER, Millipore). Cells were kept in a serum-free medium on the apical side and complete medium on the basolateral side for a further two weeks to induce differentiation and structural configuration similar to physiological conditions. The cells were used after 21 days of culture, that is to say when they are contiguous and polarized. On D20, the culture media were replaced with identical media without phenol red. On D21, the apical medium was replaced with 500 µl of test medium described in section 2.1, while the basolateral medium was replaced with 1.5 mL complete medium. The treatment was stopped after 3 or 6 hours of incubation by collection of the different media (apical, basolateral). Finally the cellular layer was washed with PBS before being scraped from the insert and collected in 500 µl PBS. Samples were kept frozen (-80 °C) until analysis.

The extraction protocol is adapted from Mercke-Odeberg *et al*(2003). Briefly, 6 volumes of hexane and 6 volumes of acetone were added, the sample vortexed for 1 min before centrifugation for 5 min at 2500 rpm, room temperature. The organic phase was transferred to a new tube and evaporated under nitrogen flux. The astaxanthin pellet was recovered in 200 µL acetone for HPLC analysis. In the case of tests on the algae source of astaxanthin, the extraction residue was taken up in 1.5 ml of acetone supplemented with 1.5 mL of cholesterol esterase solution (Sigma) in 50 mM Tris HCl pH 7 (4 U / ml) and incubated for 2 h at 37 °C, then 0.5 g of sodium decahydrate and 1 mL of petrol ether were added to the solution before centrifugation 3 min at 3500 rpm. The organic phase was transferred to a new tube containing 1 g anhydrous sodium sulfate. This step was repeated and the supernatant evaporated under a nitrogen stream. The pellet was recovered in 1.5 mL of acetone, dissolved by ultrasound for 30 sec and filtered (0.45 µm) prior to HPLC analysis.The HPLC system (Surveyor PDA ThermoFisher Scientific) was connected to a PDA detector selected to 470 nm. YMC column 30 (25 cm x 4.6 mm id; particles 5 microns; pores 100 Å) was preceded by a guard

column with the same characteristics. This system was kept at a constant temperature of 30 °C during elution (constant flow 1 ml/min). After 0.5 min of isocratic condition using 4% solvent A (ddH_2O), 15% of solvent B (MTBE) and 81% solvent C (Methanol), carotenoids were eluted over 50 min with a linear gradient of 15 to 90% of solvent B, solvent A remaining constant throughout the elution time. The column was re-equilibrated for 5 min between each analysis. Quantification was performed using a standard curve of all-trans astaxanthin (CaroteNature, Swizerland). The integration and analysis of astaxanthin peaks were performed using the ChromQuest software.

All raw data were the product of at least two independent experiments. Statistical tests were performed using SPSS v17.0 for Windows (SPSS Inc). Because of the relatively small number and heterogeneity of replicates (n=5 to 13, depending on the treatment), all statistical analyses were by default non parametric. Kruskal-Wallis tests were performed to compare the global sets of data and, when significant, post-hoc analyses were done using Dunn's pair to pair comparison.

RESULTS AND DISCUSSION

Absorption data

The absorption of astaxanthin present in the four sources was time-dependent with a median value for the unabsorbed fraction of 41.2%, 53.8%, 48.5%, 35.6% after 3 hours of treatment and 15.6%, 44.2%, 2.6%, 6.7% after 6 hours of treatmentfor the yeast, algae, bacterium and synthetic sources, respectively (Fig. 1). Although a lower rate of absorption is observed at 3 hours between the esterified and free forms, the difference is only significant between algae and synthetic astaxanthin ($p<0.05$). At 6 hours, however, the difference is statistically highly significant against all three non-esterified sources ($p<0.001$ for all three forms).

As a control molecule, beta-carotene had an unabsorbed fraction of 31.9% at 3 hours and was fully absorbed after 6 hours. This increased rate of absorption is not significant at 3 hours compared to astaxanthin but is significant at 6 hours against yeast ($p<0.01$) and algae ($p<0.001$) astaxanthin but not the two other sources.Therefore even if the transport mechanism is via passive transfer, the rate of transfer is different.

Caco2/TC7 cells were culture for 21 days on transwells prior to treatment. Astaxanthin (5µM) were added on the apical medium (DMEM serum free) and the apical, basal (DMED 10% serum) and cell fractions were collected after 3 or 6 hours. Carotenoids were quantified and expressed as percentage of unabsorbed molecule (apical). All datapoints are presented on the scatterplot for yeast, algae, bacterium and synthetic sources, in order. Dunn's pair-to-pair comparisons were performed for statistical significance. * $p<0.05$; *** $p<0.0005$

Over a third of astaxanthin remained unabsorbed (44.2%) after 6 hours for the algae source (Fig. 1). As the percentage of unabsorbed astaxanthin is not greatly improved between 3 and 6 hours of treatment (non-significant), the chromatograms confirm that only esterified astaxanthin remains in the apical medium while

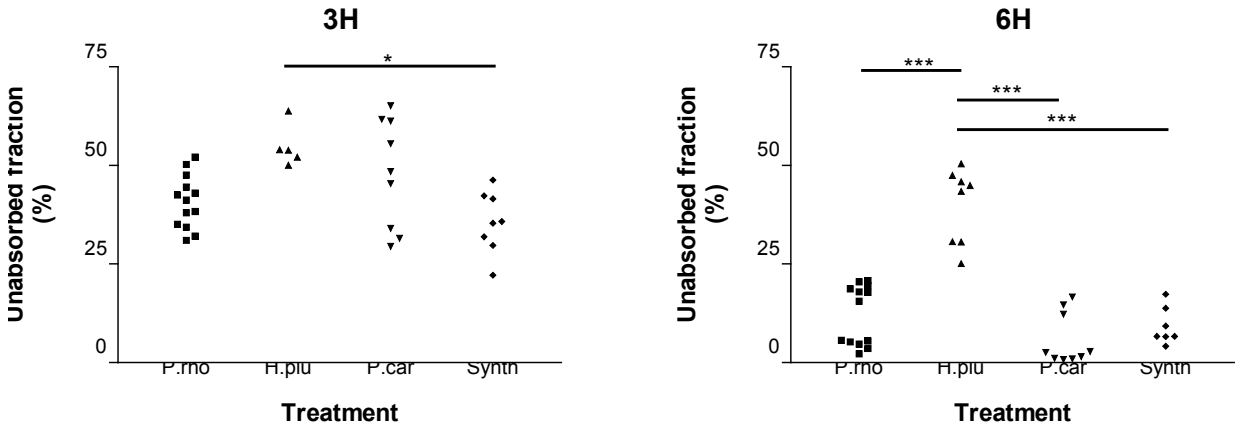

Fig. 1. Unabsorbed fractions of astaxanthin isoforms after 3 and 6 hours incubation

only free form is present in the cells (data not shown). This suggests a role for hydrolysis of astaxanthin esters leading to delayed absorption of the molecule.

Transfer to the basolateral side was very poor for astaxanthin (up to 2%, whatever the source) while it was fast and complete for beta-carotene (Tab.2). This difference is highly significant ($p<0.001$) against all sources of astaxanthin at both 3 and 6 hours of treatment. From the different sources of astaxanthin, there is no molecule detected in the basolateral compartment with algae and the transfer through the basolateral membrane seems to be time-dependent for yeast and synthetic astaxanthin, although very slow, but not for the bacterial astaxanthin. The question remains as to why the astaxanthin accumulates in the cells rather than being exported to the basolateral medium in a similar manner to beta-carotene. This may suggest the implication of an active transport system.

There are very few investigations on the bioavailability of astaxanthin reported. The main reason may be that records of astaxanthin used as a bioactive molecule in human nutrition are no more than a couple of decades old. As a consequence, studies of this carotenoid are relatively sparse. In addition, investigations on the bioavailability represent only a very small part of the available publications, which are mostly on physiological benefits. It may be as well due to the highly competitive market leading to preclinical research remaining confidential. However it is possible to compare our results with those published on other carotenoids.

O'Sullivan *et al.* (2004) showed that astaxanthin was better accumulated into Caco-2 cells over a 24-hours period compared to beta-carotene, which is in contradiction with our observations. However cells were cultured as monolayer ratherthan on a transwell system and the delivery matrix was Tween40/80 rather than biliary micelles. In addition, cells were treated with a mixture of carotenoids rather than individual compound, which may impact the absorption rate of individual molecules. A similar result with higher cellular uptake and secretion to the basolateral side was observed on a transwell culture set-up when comparing astaxanthin to beta-carotene presented on the apical side in Tween40 with chylomicron-stimulating molecules for 16 hours (O'Sullivan, Ryan & O'Brien, 2007). Sy *et al.* (2012) did not find any carotenoid into the basolateral medium after a 3 hours incubation in the apical side with natural or synthetic micelles. This is consistent with our observations for astaxanthin (less than 2% in the basolateral fraction) but not beta-carotene. All experimental conditions were similar between the two set-ups. Our data also showed a higher transfer rate to the cellular fraction at 3 hours (around 50%) compared to the authors (10%) but all agree on a similar uptake rate for the two carotenoids at 3 hours. The higher uptake rate should not be due to adsorbed carotenoids as the washing step was performed in our experiments as well.

The rate of absorption measured was increased for the free form compared to esterified astaxanthin from algae. This is consistent with the data reported by Lyons *et al.* (2002), where cellular incorporation from synthetic astaxanthin was nearly twice higher than algae astaxanthin. Esterification

Tab.2. Absorption of astaxanthin and beta-carotene by Caco2/TC7 cells

Source	3h				6h			
	% Absorbed (BL+C)		of which in BL		% Absorbed (BL+C)		of which in BL	
P.rho	59.22 (6.75)	*b*	1.01 (0.50)	*b*	87.83 (7.54)	b	2.20 (1.69)	*b*
P.car	51.94 (13.83)	*ab*	1.19 (2.08)	*b*	94.08 (6.58)	c	0.75 (1.58)	*a*
H.plu	45.26 (5.29)	*a*	0.00 (0.00)	*a*	60.20 (9.50)	a	0.00 (0.00)	*a*
Synth	64.38 (7.78)	*b*	1.00 (0.95)	*b*	90.78 (4.70)	bc	2.54 (1.70)	*b*
B-car	60.32 (15.67)	*b*	55.49 (13.91)	*c*	100.00 (0.00)	d	95.87 (6.52)	*c*

Caco2/TC7 cells were culture for 21 days on transwells prior to treatment. Astaxanthin or beta-carotene (5µM) were added on the apical medium (DMEM serum free) and the apical, basal (DMED 10% serum) and cell fractions were collected after 3 or 6 hours. Carotenoids were quantified and expressed as percentage of absorbed molecule (cell + basolateral). Data are expressed as mean and standard deviation and different letters in columns indicate a significant difference between the treatments (Kruskall-Wallis followed by Dunn's pair-to-pair comparisons).

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